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An Improved Method for the Measurement of Urinary and Plasma F_2-Isoprostanes Using Gas Chromatography–Mass Spectrometry

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We have developed an improved method for the measurement of F_2-isoprostanes using stable isotope dilution capillary gas chromatography/electron capture negative ionization mass spectrometry (GC-ECNI-MS). The F_2-isoprostane family consists of a series of chemically stable prostaglandin F_2 (PGF_2)-like compounds generated during peroxidation of arachidonic acid in phospholipids. There is evidence that measurement of F_2-isoprostanes represents a reliable and useful index of lipid peroxidation and oxidant stress *in vivo*. Furthermore, 8-epi-$PGF_{2\alpha}$, which is one of the more abundant F_2-isoprostanes, is biologically active, being a potent mitogen and vasoconstrictor of rat and rabbit lung and kidney, as well as a partial agonist of platelet aggregation. Measurement of F_2-isoprostanes in biological samples is complex and has involved methods which utilize multiple chromatographic steps, including separation by thin-layer chromatography, leading to poor sample recovery. We now present an improved method for the measurement of plasma and urinary F_2-isoprostanes using a combination of silica and reverse-phase extraction cartridges, high-performance liquid chromatography (HPLC), and GC-ECNI-MS. Different approaches to the derivatization of the F_2-isoprostanes prior to GC-ECNI-MS are also addressed. The overall recovery of F_2-isoprostanes is improved (approx 70% for urine) and the within and between assay reproducibility is 6.7% (n = 23) and 3.7% (n = 3), respectively. The mean urinary excretion of F_2-isoprostanes in eight healthy males was 365 ± 5 pmol/mmol creatinine and in three smokers 981 ± 138 pmol/mmol creatinine. The mean total (free + esterified) plasma F_2-isoprostane concentration was 952 ± 38 pmol/liter, with a within and between assay reproducibility of 8% (n = 13) and 5.6% (n = 3), respectively. This improved method for the measurement of F_2-isoprostanes represents a significant advance in terms of the rapidity and yield in the purification of biological samples. The inclusion of HPLC separation enables improved analysis of F_2-isoprostanes by GC-MS. This methodology will assist in defining the role of F_2-isoprostanes as *in vivo* markers of oxidant stress in clinical and experimental settings.

Key Words: **F_2-isoprostanes; 8-iso-prostaglandin $F_{2\alpha}$; gas chromatography–mass spectrometry; lipid peroxidation; urine; plasma; free radicals.**

Recently, considerable attention has focused on free radicals and their possible role in a variety of disease processes (1–3). However, much of the evidence is indirect and circumstantial, due primarily to limitations of available indices of free radical-catalyzed events *in vivo*. Measures of lipid peroxidation are often employed to implicate free radicals in pathophysiological processes. These have traditionally involved the use of assays directed against nonspecific or unstable metabolites such as short-chain alkanes, malondialdehyde, lipid hydroperoxides, or *ex vivo* induced lipoprotein oxidizability (2, 3).

The recently discovered F_2-isoprostanes consist of a series of chemically stable prostaglandin F_2-like compounds generated during peroxidation of unsaturated fatty acids (primarily arachidonic acid) in membrane phospholipids (4–7). These compounds are primarily, but not exclusively, formed by a mechanism independent of the cyclooxygenase pathway (6, 7). There is substantial evidence that quantitation of F_2-isoprostanes represents a reliable and useful assessment of

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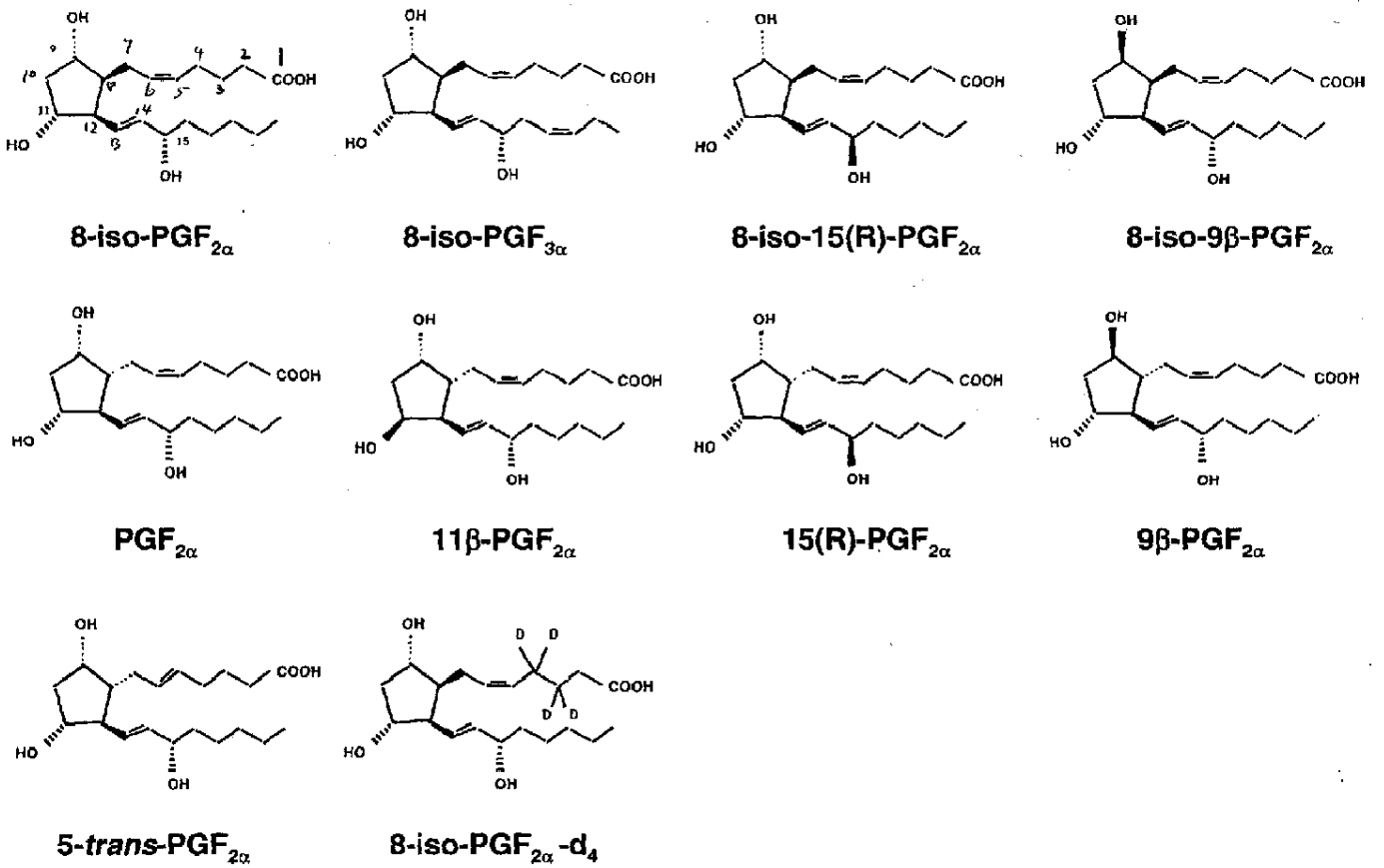

FIG. 1. (Top) 8-Iso-$PGF_{2\alpha}$, 8-iso-$PGF_{3\alpha}$ (derived from EPA), the epimeric 8-iso-15(R)-$PGF_{2\alpha}$ and 8-iso-9β-$PGF_{2\alpha}$ analogues. (Middle) $PGF_{2\alpha}$ and epimers 11β-$PGF_{2\alpha}$, 15(R)-$PGF_{2\alpha}$, and 9β-$PGF_{2\alpha}$. (Bottom) 5-*trans*-$PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$-d_4.

lipid peroxidation and oxidant stress *in vivo* (4, 6, 7). One of these compounds, 8-isoprostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$)[2] (Fig. 1), is formed *in vivo* (8). It is biologically active, being a potent vasoconstrictor of rat and rabbit lung and kidney (4, 9–11), actions shown to be mediated at least in part via interactions with the thromboxane A_2 receptor (9). However, evidence also exists for a separate isoprostane receptor that is distinct from the thromboxane receptor (12). 8-Iso-$PGF_{2\alpha}$ is a mitogen in 3T3 cells and in vascular smooth muscle cells (13). 8-Iso-$PGF_{2\alpha}$ also acts as a partial agonist on platelet aggregation, inducing platelet shape change (14). Elevated levels of F_2-isoprostanes have been reported in animal models of free radical injury (15), under human conditions associated with increased oxidant stress (16–25), and in *in vitro* experimental models (21, 26–28).

Measurement of F_2-isoprostanes in biological samples is difficult because they must be separated from other isoprostanes, prostaglandins, and their metabolites. The methods utilized thus far have involved multiple chromatographic steps (29). These have generally included separation on silica and reverse-phase cartridges, as well as thin-layer chromatography (TLC), the latter notorious as both time-consuming and leading to poor sample recovery. The most reliable, sensitive, and specific method for the measurement of F_2-isoprostanes involves using a stable isotope dilution assay utilizing capillary gas chromatography/electron capture negative ionization mass spectrometry (GC-ECNI-MS). Herein, we now present an improved method for the measurement of plasma and urinary F_2-isoprostanes using a combination of silica and reverse-phase cartridges, high-performance liquid chromatography (HPLC), and GC-ECNI-MS.

[2] Abbreviations used: PGF_2, prostaglandin F_2; ECNI, electron capture negative ionization; BSTFA, *N,O-bis*-(trimethylsilyl)trifluoroacetamide; TMCS, trimethylchlorosilane; MTBSTFA, *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide; TBDMCS, *tert*-butyldimethylchlorosilane; PFBBr, pentafluorobenzylbromide; DIPEA, *N,N*-diisopropylethylamine; TBDMS, *tert*-butyldimethylsilyl; TMS, trimethylsilyl.

MATERIALS AND METHODS

Chemicals, Reagents, and Chromatography

Synthetic prostaglandins, including 8-iso-$PGF_{2\alpha}$, 8-iso-$PGF_{3\alpha}$, 8-iso-15(R)-$PGF_{2\alpha}$, 8-iso-9β-$PGF_{2\alpha}$, $PGF_{2\alpha}$, 11β-$PGF_{2\alpha}$, 15(R)-$PGF_{2\alpha}$, 9β-$PGF_{2\alpha}$, 5-*trans*-$PGF_{2\alpha}$, and 8-iso-$PGF_{2\alpha}$-d_4, were purchased from Cayman Chemicals (Ann Arbor, MI) and used without further purification. All solvents were of HPLC grade. The silylating agent *N,O-bis*-(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane (BSTFA + TMCS, 99:1) was purchased from Pierce Chemicals (Rockford, IL); *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA) and MTBSTFA + 1% *tert*-butyldimethylchlorosilane (MTBSTFA + TBDMCS, 99:1) were purchased from Aldrich Chemicals (Milwaukee, WI). Pentafluorobenzylbromide (PFBBr) and *N,N*-diisopropylethylamine (DIPEA) were purchased from Sigma Chemicals (St Loius, MO). C_{18} and silica Sep-Pak Cartridges were from Waters Associates (Milford, MA).

Urine and Blood Collection from Humans

Eight healthy male nonsmokers aged 37.6 ± 3.4 years and three healthy smokers (two females and one male) aged 33.3 ± 1.7 years were recruited from the general community. Urine from a 24-h collection was stored at −80°C without preservative. Blood was collected into ice-cold tubes containing EDTA (1 mg/ml) and reduced glutathione (1 mg/ml). Blood samples were centrifuged at 1500*g* for 10 min at 4°C and the plasma, stored at −80°C in 2-ml aliquots, was protected from oxidation with butylated hydroxy toluene (200 μg/ml) added as an antioxidant. The study was approved by the ethics committee of the Royal Perth Hospital and all subjects gave written consent. All procedures followed were in accordance with institutional guidelines.

Analysis of Urinary F_2-Isoprostanes

Purification. Urine from a 24-h collection, stored at −80°C without preservative, was allowed to thaw. An aliquot (2 ml) was acidified to pH 3 with 2 M HCl and 8-iso-$PGF_{2\alpha}$-d_4 (5 ng, internal standard) was added. The mixture was vortexed and applied to a C_{18} Sep-Pak Cartridge preconditioned with methanol (5 ml), followed by water (pH 3, 5 ml). The sample and subsequent solvents were eluted through the column using a 10-ml plastic syringe. Using a 12-port vacuum manifold (Alltech, Deerfield, IL) attached to a vacuum pump (Dynavac, Melbourne, Australia) enabled the simultaneous extraction of 12 samples. The column was washed sequentially with water (pH 3, 10 ml), acetonitrile/water (15:85, 10 ml), and petroleum ether (10 ml), and the F_2-isoprostanes were then eluted with ethyl acetate/petroleum ether (50:50, 10 ml). The eluate was dried over anhydrous $MgSO_4$ and then applied to a silica Sep-Pak Cartridge. The column was washed with ethyl acetate (5 ml) and the F_2-isoprostanes were eluted with ethyl acetate/methanol (50:50, 5 ml). The eluate was evaporated to dryness in a SpeedVac SVC200 Concentrator (Savant Instruments, Farmingdale, NY) attached to a Refrigerated RVT4104 Vapor Trap (Savant Instruments).

The extract was reconstituted in methanol (40 μl) and subjected to chromatography on a LiChrospher 100 RP-18, 125 × 4-mm, 5-μm column (Hewlett–Packard, Waldbronn, Germany) using a Hewlett–Packard Series 1100 HPLC System (Hewlett–Packard) connected to a Gilson FC-205 Fraction Collector (Gilson Inc., Middleton, WI). The mobile phase consisted of acetonitrile (solvent A) and 0.05% acetic acid in water (solvent B). Separation was carried out with a linear gradient starting with 10% solvent A and ramping to 50% solvent A at 20 min and then to 100% solvent A at 25 min and maintained for a further 5 min. The total run time was 30 min. The flow rate was 1 ml/min and absorbance was measured at 205 nm. A fraction collected between 14.8 and 15.8 min contained the F_2-isoprostanes which eluted at approximately 15 min. The eluate from the HPLC was evaporated to dryness in a SpeedVac SVC200 Concentrator.

Derivatization for gas chromatography. The F_2-isoprostane fraction was treated with a mixture of PFBBr (40 μl, 10% (v/v) in acetonitrile) and DIPEA (20 μl, 10% (v/v) in acetonitrile) at room temperature for 30 min. The reagents were dried under N_2 to give the F_2-isoprostane pentafluorobenzyl esters. The sample was then treated with BSTFA + TMCS (99:1, 20 μl) and anhydrous pyridine (10 μl) at 45°C for 20 min to yield the trimethylsilylethers.

Gas chromatography–mass spectrometry. For analysis of F_2-isoprostanes, samples were reconstituted in isooctane (25 μl) and analyzed on a Hewlett–Packard HP 5890 Series II Plus gas chromatograph (Hewlett-Packard, Palo Alto, CA), coupled to an HP 5989B Mass Spectrometer (Hewlett-Packard) and using HP ChemStation G1034C Revision C.03.03 Software. Samples were chromatographed on an HP-5MS (crosslinked 5% diphenyl and 95% dimethylpolysiloxane) column (30 m × 0.25 mm, 0.25 μm film thickness, Hewlett-Packard) using helium as the carrier gas at a flow rate of 0.68 ml/min. ECNI used methane as the reagent gas at an ion source pressure of 1.8 torr. The injector temperature was maintained at 250°C, the transfer line at 280°C, and the ion source and quadrapole temperatures were 200 and 100°C, respectively. Injections were made using an HP 6890 Series Injector autosampler (Hewlett–Packard) in a splitless mode for the first 1 min. The initial column temperature of 160°C was held for 1 min and then programmed from 160 to 300°C at

20°C/min and maintained at 300°C for 17 min, giving a total run time of 25 min.

The mass spectrometer was operated in the selective ion monitoring (SIM) mode and collected data between 10 and 13 min of each program. The F_2-isoprostanes were detected by monitoring *m/z* 569, which corresponded to the loss of the pentafluorobenzyl radical from the intact F_2-isoprostane anion (M-181). The corresponding ion for the deuterium-labeled 8-iso-$PGF_{2\alpha}$-d_4 was *m/z* 573 and that for 8-iso-$PGF_{3\alpha}$ was *m/z* 567. Peak identification was based on comparison of retention times with standards.

Analysis of Plasma F_2-Isoprostanes

To 2 ml of plasma in a glass tube were added 8-iso-$PGF_{2\alpha}$-d_4 (2 ng, internal standard) and 1 M KOH in methanol (1 ml). The tube was flushed with N_2 and heated at 40 °C for 30 min. The mixture was cooled, diluted with methanol (1 ml), and centrifuged at 1500*g* for 10 min at 4°C to precipitate proteins. The supernatent was diluted with 0.1 M phosphate buffer pH 4 (8 ml), adjusted to pH 3 with 2M HCl, and centrifuged at 1500*g* for 10 min at 4°C to remove any remaining protein precipitate. The hydrolysate was applied to a C_{18} Sep-Pak Cartridge and chromatographed using the same procedure outlined above for urine.

Derivatization of 8-Iso-$PGF_{2\alpha}$-Methyl Ester

The 8-iso-$PGF_{2\alpha}$ was treated with diazomethane to afford the methyl ester in quantitative yield. In separate experiments, aliquots of the 8-iso-$PGF_{2\alpha}$ - methyl ester (1 μg) were treated with MTBSTFA + TBDMCS (99:1, 20 μl) and anhydrous pyridine (10 μl) for 18, 24, 42, 48, and 66 h. Experiments were carried out at room temperature, 45, 55, and 65°C. Samples were reconstituted in isooctane (25 μl) and analyzed by GC-MS. The mass spectrometer was operated in the electron impact scan mode, and mass chromatograms were recorded between 10 and 14 min in the mass range 50–800 amu. The peaks detected were the 8-iso-$PGF_{2\alpha}$ - methyl ester with one, two, or three *tert*-butyldimethylsilyl (TBDMS) derivatives on the three hydroxyl groups of the molecule.

RESULTS

Separation of F_2-Isoprostanes

The overall recovery of F_2-isoprostanes from urine was approximately 70% and represents a major improvement over previous methods which have utilized TLC. The F_2-isoprostane fraction was evaporated soon after elution, as prolonged standing in the mobile solvent which contains 0.05% acetic acid was shown to lead to poor recovery. Plasma determination of F_2-isoprostanes entailed mild base hydrolysis of samples prior to chromatography.

Derivatization of 8-Iso-$PGF_{2\alpha}$ to the TMS- and TBDMS-Ethers

Standard 8-iso-$PGF_{2\alpha}$ was used in experiments designed to examine the derivatization of F_2-isoprostanes prior to GC-MS. 8-Iso-$PGF_{2\alpha}$ was converted to the methyl ester and further derivatized to either the trimethylsilyl (TMS)- or the TBDMS-ethers. Formation of the TMS-ethers was afforded in quantitative yield by treatment of samples with BSTFA + TMCS (99:1) and anhydrous pyridine at 45°C for 20 min. The TBDMS-ethers, however, were not detected after reaction with MTBSTFA + TBDMCS (99:1) under these conditions. Although the yield of the TBDMS-ethers was improved with increased reaction temperature and prolonged reaction time, there was poor overall recovery of product due to substantial decomposition. Use of MTBSTFA afforded substantially reduced yield compared to the same reagent containing 1% TBDMCS. Therefore, limitations with use of this derivative were the relatively low yields with moderate reaction temperature, the inconvenience of long reaction times, and decomposition of product at elevated temperature.

Gas Chromatography - Mass Spectrometry

Gas chromatographic separation of 8-iso-$PGF_{2\alpha}$, the epimeric 8-iso-15(R)-$PGF_{2\alpha}$ and 8-iso-9β-$PGF_{2\alpha}$ analogues, 8-iso-$PGF_{3\alpha}$ (derived from eicosapentaenoic acid), 5-*trans*-$PGF_{2\alpha}$, $PGF_{2\alpha}$, and the 11β-, 9β- and 15(R)-epimers of $PGF_{2\alpha}$ was carried out after conversion to the pentafluorobenzyl-ester and TMS-ether derivatives (Fig. 2). The carboxylate anion *m/z* 569 was used in SIM to detect the elution of all but 8-iso-$PGF_{3\alpha}$, which was detected at *m/z* 567. Under these conditions, 8-iso-$PGF_{2\alpha}$ eluted at 11.15 min and could not be separated from 8-iso-15(R)- $PGF_{2\alpha}$ (11.12 min) and 9β-$PGF_{2\alpha}$ (11.13 min). 8-Iso-9β-$PGF_{2\alpha}$ eluted at 11.21 min and was partially resolved from 8-iso-$PGF_{3\alpha}$ (11.24 min). $PGF_{2\alpha}$ eluted at 11.54 min and was partially separated from its two epimeric isomers, 11β-$PGF_{2\alpha}$ (11.43 min) and 15(R)-$PGF_{2\alpha}$ (11.49 min). The 8-iso-$PGF_{2\alpha}$-d_4 eluted at 11.14 min (not shown in Fig. 2) and was detected at *m/z* 573. The retention times of 8-iso-$PGF_{2\alpha}$. 8-iso-$PGF_{3\alpha}$, and 8-iso-$PGF_{2\alpha}$-d_4 on the GC were highly reproducible with a coefficeint of variation of 0.03, 0.03, and 0.07%, respectively. The limit of detection was 250 fg/μl for a signal to noise ratio of 12:1 in the SIM mode.

Human Urine and Plasma F_2-Isoprostanes

Analysis of the urine and plasma samples revealed a series of peaks representing F_2-isoprostanes. Typical

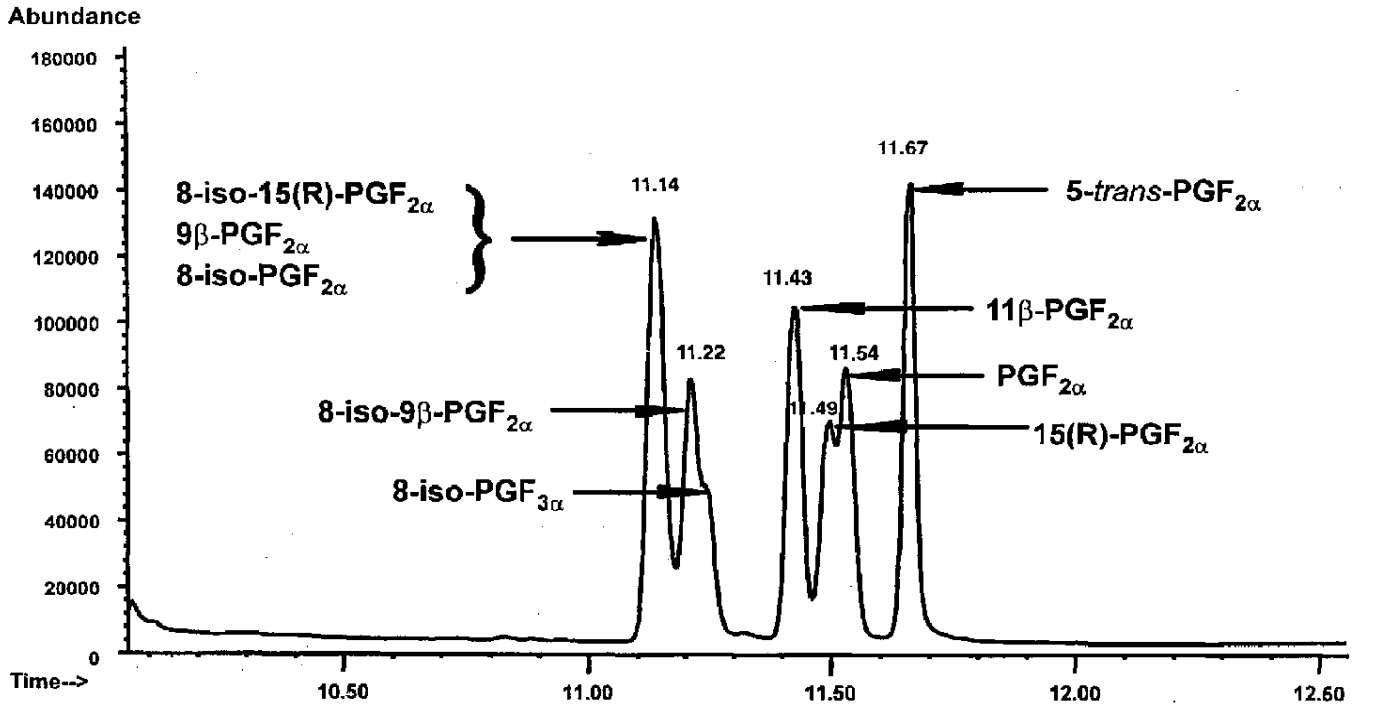

FIG. 2. GC-MS analysis with electron capture negative ionization of the pentafluorobenzyl trimethylsilyl ether derivatives of a mixture of 8-iso-$PGF_{2\alpha}$, 9β-$PGF_{2\alpha}$, 8-iso-15(R)-$PGF_{2\alpha}$, 8-iso-9β-$PGF_{2\alpha}$, 8-iso-$PGF_{3\alpha}$, 11β-$PGF_{2\alpha}$, 15(R)-$PGF_{2\alpha}$, $PGF_{2\alpha}$, and 5-*trans*-$PGF_{2\alpha}$ (2 ng of each injected). The carboxylate anion at *m/z* 569 was used for all but 8-iso-$PGF_{3\alpha}$ (*m/z* 567) in SIM mode to detect the elution of the components individually or as a mixture. See Materials Methods for the conditions used. Retention times are indicated above each peak.

chromatograms are shown in Figs. 3 and 4 respectively. The mean urinary excretion of F_2-isoprostanes was 365 ± 5 pmol/mmol creatinine (n = 8) (range, 326–414 pmol/mmol creatinine) and the mean plasma concentration was 952 ± 38 pmol/liter (n = 8) (range, 703–1087 pmol/liter). The within and between assay reproducibility was 6.7% (n = 23) and 3.7% (n = 3), respectively, for urinary F_2-isoprostanes, and 8.0% (n = 13) and 5.6% (n = 3), respectively, for plasma F_2-isoprostanes. The urinary F_2-isoprostane level in the three smokers was 981 ± 138 pmol/mmol creatinine (range, 759–1233 pmol/mmol creatinine) which was significantly increased compared with nonsmokers.

DISCUSSION

The extraction and purification of F_2-isoprostanes from urine or plasma are a prerequisite to their analysis because of the interference by structurally related metabolites. We have refined the method initially described by Morrow *et al.* (29). Samples were first extracted on a reverse-phase cartridge followed by silica cartridge chromatography. Although previous methods have relied on one or two TLC steps for further purification, both before and after derivatization to the pentafluorobenzyl ester (29), we introduced an HPLC purification step which provides a more effective clean-up and improved yield prior to GC/MS. One further advantage of HPLC is that it is easily automated by the use of an autoinjector and autosampler. Thin-layer chromatography of F_2-isoprostanes has been associated with a number of methodological problems. It is notorious for being both time-consuming and it leads to poor sample recovery (typically 30–40%). Furthermore, in the absence of radiolabeled F_2-isoprostane standards, TLC has required cochromatography of 8-iso-$PGF_{2\alpha}$ or $PGF_{2\alpha}$ for the localization of F_2-isoprostanes being measured in biological samples. It has also required that these standards are chromatographed on a separate TLC plate in order to avoid any cross-contamination of the samples (29).

The measurement of F_2-isoprostanes presents a new means of assessing *in vivo* lipid peroxidation and oxidative stress (4–7). In support of this, there are now a number of reports in humans demonstrating elevated levels of F_2-isoprostanes in situations associated with increased oxidative stress. Furthermore, it has been proposed that the measurement of F_2-isoprostanes may serve as a useful noninvasive marker of the initial events associated with lipid peroxidation, rather than the measurement of short-chain alkanes such as 4-hydroxynonenal or malondialdehyde (2, 7, 29). More re-

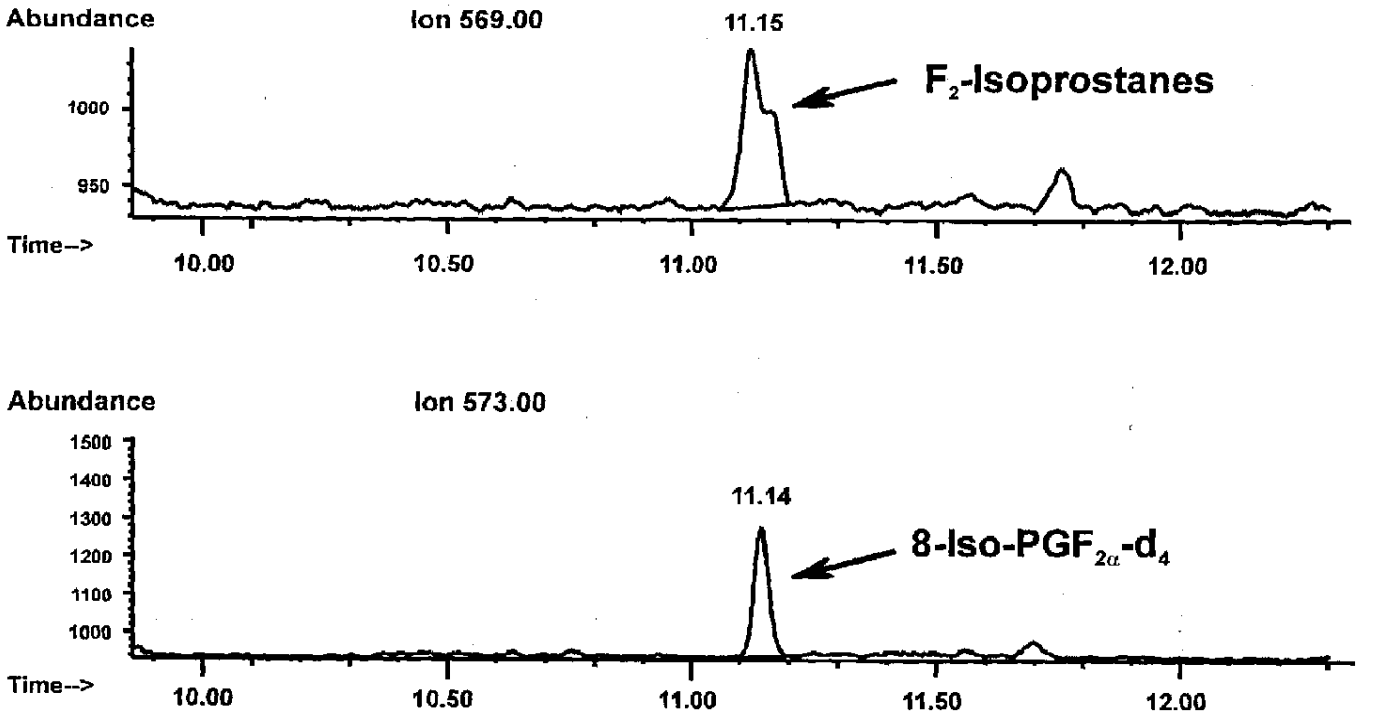

FIG. 3. Typical chromatogram of urinary F_2-isoprostanes analyzed by GC-MS with ECNI detection in the SIM mode. The upper trace shows ions *m/z* 569, with the F_2-isoprostanes eluting at 11.15 min. The lower trace shows the peak of the deuterated internal standard 8-iso-$PGF_{2\alpha}$-d_4 monitored at *m/z* 573 elutes at 11.14 min.

cent evidence supports the formation of F_3-isoprostanes from the marine ω3 fatty acid eicosapentaenoic acid. Nourooz-Zadeh *et al.* showed that F_3-isoprostanes were formed during the *in vitro* peroxidation of EPA-enriched liposomes with the peroxyl radical generator AAPH or copper ions (30).

Elevated levels of F_2-isoprostanes have been reported in animal models of free radical injury (15), in humans after poisoning (16), in smokers (17, 18), non-insulin-dependent diabetics (19), and in patients with liver cirrhosis (20). Increased urinary F_2-isoprostanes have been observed after carotid reperfusion in patients undergoing endarterectomy (21), in patients undergoing angioplasty (22), as well as in patients with acute myocardial infarction treated with thrombolytic drugs and after clamp release while undergoing coronary artery bypass surgery (23). Increased plasma and urinary F_2-isoprostanes were also shown in pregnant women with preeclampsia (24). It has been suggested that F_2-isoprostanes may reflect lipid peroxidation in atherogenesis. In support of this, there is a marked augmentation of F_2-isoprostanes formation in zymosan-stimulated human monocytes co-incubated *in vitro* with LDL (26). Consistent with this hypothesis, F_2-isoprostanes have been shown to be elevated in hypercholesterolaemic patients (25) and have been detected both in oxidized LDL (27) and in human atherosclerotic plaque (21, 28).

There are recent reports that F_2-isoprostanes, are at least in part, also formed via the cyclooxygenases. This has raised concern regarding the specificity of measuring F_2-isoprostanes as a marker of nonenzymatic lipid peroxidation. Wang *et al.* reported that although urinary 8-iso-$PGF_{2\alpha}$ excretion was not altered following iboprofen or aspirin administration to healthy men and women, serum levels were suppressed after aspirin (31). Schweer *et al.* also showed a small but significant attenuation of urinary F_2-isoprostanes and 8-iso-$PGF_{2\alpha}$ excretion by indomethacin in humans (32). 8-Iso-$PGF_{2\alpha}$ has been reported as a minor product of cyclooxygenases-1 and -2 from microsomes isolated from ram seminal vesicles (33) and human platelets (34). Furthermore, induction of cyclooxygenase in human monocytes is associated with 8-iso-$PGF_{2\alpha}$ formation, which can be suppressed with selective inhibitors of the enzyme (26, 35). Recently, Klein *et al.* have also shown that mesangial cells in culture generate 8-iso-$PGF_{2\alpha}$ following interleukin-1 stimulation (36). These findings, however, must be viewed together with those of other reports in which 8-iso-$PGF_{2\alpha}$ formation was not reduced following administration of cyclooxygenase inhibitors (4, 25, 37). In addition, Wang *et al.* have suggested that

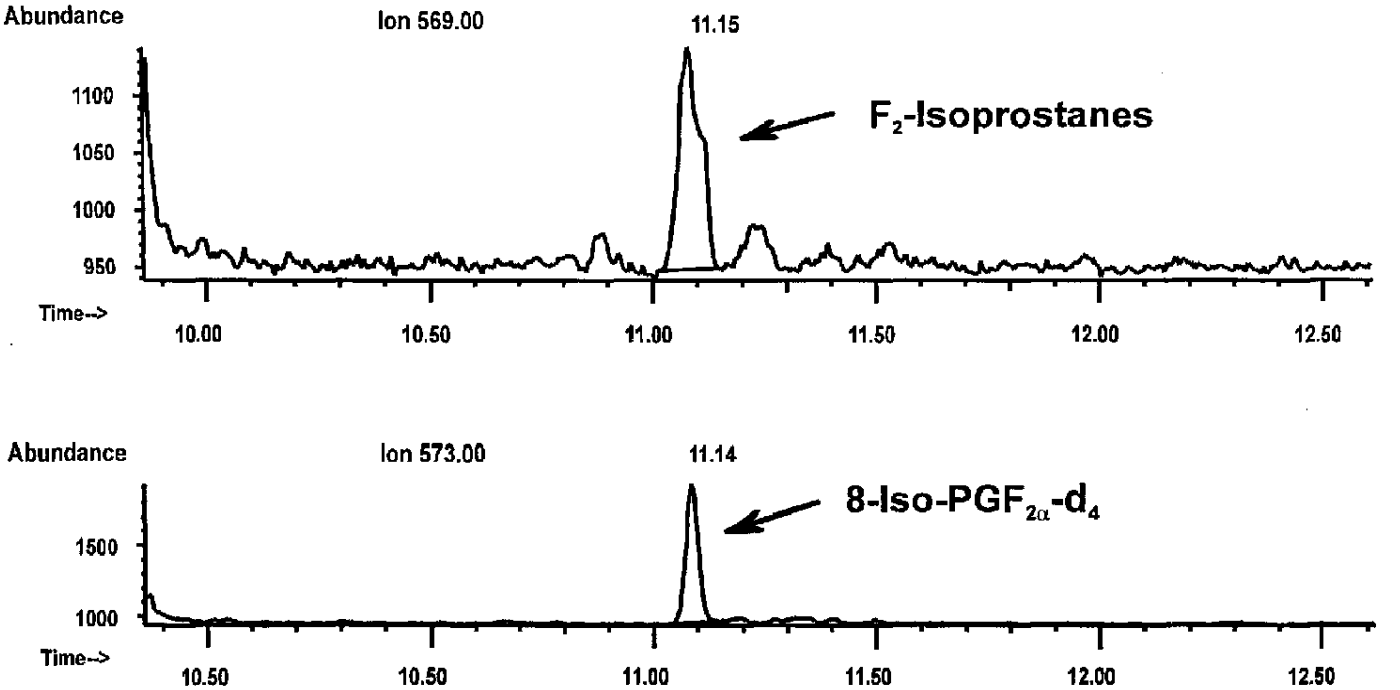

FIG. 4. Typical chromatogram of plasma total F_2-isoprostanes analyzed by GC-MS with ECNI detection in the SIM mode. The upper trace shows ions *m/z* 569, with the F_2-isoprostanes eluting at 11.15 min. The lower trace shows the peak of the deuterated internal standard 8-iso-$PGF_{2\alpha}$-d_4 monitored at *m/z* 573 elutes at 11.14 min.

enzymatic mechanisms of isoprostane biosynthesis do not contribute importantly to the global biosynthesis of these metabolites *in vivo* under physiological circumstances (31).

Various methodologies using gas chromatographic-mass spectrometric techniques (4, 5, 8, 9, 15, 31, 34, reviewed in 29), immunoassays (31), radioimmunoassay (38), and combined immunoaffinity extraction/mass spectrometry (39) have been employed to analyze F_2-isoprostanes. In view of the complexity of the mixture of F_2-isoprostanes in biological samples, GC-MS has been recognized as the definitive method of measurement to date and ECNI has been instrumental in the identification of F_2-isoprostanes. This methodology uses the carboxylate anion at *m/z* 569 to detect the elution of the isomeric F_2-isoprostanes from the GC column (29). More recently, electrospray ionization ESI coupled with liquid chromatography tandem mass spectrometry (LC/MS/MS) has been used to specifically detect F_2-isoprostanes generated *in vitro* by free radical oxidation of arachidonic acid (40). This technology was further used to identify the F_2-isoprostane regioisomers in the livers of rats treated with carbon tetrachloride (41). In a subsequent report, Schweer *et al.* have used gas chromatographic/triple-stage quadrupole mass spectrometry (GC/MS/MS) to identify F_2-isoprostanes and 8-iso-$PGF_{2\alpha}$ in human urine (32). There is no question that the use of LC/MS/MS and GC/MS/MS affords the highest selectivity and sensitivity of all techniques for the measurement of individual F_2-isoprostane isomers. Indeed, as there are theoretically 64 different F_2-isoprostanes possible from a free radical mechanism of oxidation (6), these studies will continue to provide invaluable information as to which isomers are formed and which ones are the most important under different conditions. Unfortunately, however, few laboratories have these highly complex and expensive instruments available. Therefore, it was our aim to develop and improve current published methods for measuring F_2-isoprostanes in biological samples using GC-MS.

The assay we describe was initially developed for the measurement of urinary F_2-isoprostanes, but, as demonstrated, is also readily applicable to the assessment of total plasma concentration following base hydrolysis. Urinary F_2-isoprostanes are more convenient to measure in that unlike plasma, sampling is noninvasive, sample handling and storage are simpler, and there is no evidence of artifactual formation (5). In addition, we, like others (4, 5, 8, 9, 15, 29, 32), considered it more appropriate and accurate to measure total F_2-isoprostanes rather than attempt to measure levels of 8-iso-$PGF_{2\alpha}$. Although others have reported 8-iso-$PGF_{2\alpha}$ concentrations (16, 18, 19, 31), it is interesting to note that this metabolite is one of the minor components of the F_2-isoprostanes (32). Furthermore, few

studies in which 8-iso-$PGF_{2\alpha}$ levels have been reported using GC-MS have presented chromatograms for comparison. Others have shown poor resolution of the 8-iso-$PGF_{2\alpha}$ peak from other peaks using GC-MS with ECNI detection (19).

The derivatization of the F_2-isoprostanes has not been previously examined in detail. GC-MS with ECNI detection requires initial formation of the pentafluorobenzyl ester and further derivatization of the three hydroxyl groups on the molecule. The TMS-ether has been the preferred derivative for GC-MS analysis (4, 5, 8, 9, 15, 29), although the TBDMS-ether has been reported (16, 18, 31). We found that there was poor conversion of 8-iso-$PGF_{2\alpha}$ to the TBDMS-ether at room temperature. Although the yield was improved with prolonged reaction time or increased reaction temperature, this was found to be impracticable. Furthermore, increasing the temperature was accompanied by decomposition of product.

Separation of most epimers of $PGF_{2\alpha}$ and F_2-isoprostanes was achievable under the GC-MS conditions employed. However, 8-iso-$PGF_{2\alpha}$ coeluted with one of the $PGF_{2\alpha}$ epimers (9β -$PGF_{2\alpha}$) as well as 8-iso-15(R)-$PGF_{2\alpha}$. Unfortunately the lack of availability of other F_2-isoprostane epimers precluded the determination of the retention times of these metabolites. Nonetheless, coelution of 8-iso-$PGF_{2\alpha}$ with other F_2-isoprostanes cannot be discounted and lends further support to the determination of total F_2-isoprostanes rather than 8-iso-$PGF_{2\alpha}$ using this methodology. The basal plasma concentration and urinary excretion of F_2-isoprostanes measured by our method in healthy male nonsmokers was consistent with that reported by others using different extraction and purification methods before GC-MS (6).

In conclusion, we have established an improved method for the determination of plasma and urinary F_2-isoprostanes using a combination of reverse-phase and silica cartridge extraction, HPLC chromatography, and GC-MS with ECNI detection. This method represents a significant advance in terms of the rapidity and yield in the purification of biological samples over previously published GC-MS methods. It is envisaged this methodology will assist to better define the role of F_2-isoprostanes as an *in vivo* marker of oxidant stress in clinical and experimental settings.

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Ref 4. 5. 8. 9, 15. 29. 32. Consider more appropriate and accurate to measure total F_2-isoprostanes rather than attempt to measure levels of only 8-iso-$PGF_{2\alpha}$